Journal of Immunological Methods 443 (2017) 18–25

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Antibody-mediated depletion of immunosuppressive factors from ovarian carcinoma-associated ascites for investigation of paracrine versus autocrine effects

E. Brencicova [a,b], S.S. Diebold [a,c,*]

[a] King's College London, Peter Gorer Department of Immunobiology, Division of Immunology, Infection and Inflammatory Disease, UK
[b] University of Bern, Institute for Forensic Medicine, Switzerland
[c] The National Institute for Biological Standards and Control, Biotherapeutics Division, UK

ARTICLE INFO

Article history:
Received 9 November 2016
Received in revised form 26 January 2017
Accepted 26 January 2017
Available online 31 January 2017

Keywords:
Depletion
Antibodies
Cytokines
Beads
Autocrine
Ascites

ABSTRACT

Many studies seek to explore the impact of extrinsic soluble factors present in serum, interstitial fluids or cell-conditioned media on cells in vitro. A convenient approach to elucidate the effects of a particular factor is its selective neutralization. However, intrinsic production of soluble factors such as cytokines by the cultured cells is common and can have an impact via autocrine mechanisms. The addition of cytokine-specific neutralizing antibodies leads to neutralization of the targeted factors irrespective of their source and affects paracrine and autocrine effects alike. Thus, neutralization assays are not suitable to irrevocably demonstrate that the examined factors exert their effect via a paracrine mechanism. We were interested in investigating the impact of immunosuppressive factors present in ovarian carcinoma-associated ascites by dissecting paracrine versus autocrine effects of interleukin 10 (IL-10) and prostaglandin E2 (PGE_2) on the activation of monocyte-derived dendritic cells (DC). We explored several methods of depletion based on introduction of the neutralizing antibodies bound to beads. Here we describe the pitfalls of the investigated depletion approaches and show the importance of monitoring the presence of residual neutralizing antibodies in the sample upon depletion, which impacts on the suitability of the approach to distinguish paracrine from autocrine effects. Only one of three investigated approaches showed no dislocation of neutralizing antibody from the beads into the sample. This method, which is based on covalently linking antibody to magnetic beads harbouring a reactive group allowed for the complete removal of the investigated factors from ascites and represents an elegant tool to elucidate immunoregulatory or -stimulatory cytokine networks in considerably more depth than the use of neutralizing antibodies in cell cultures alone can contribute.

1. Introduction

Development of ascites is a hallmark symptom of the late stages of ovarian carcinoma, and soluble tumour-associated factors within can affect disease progression and interfere with anti-tumour vaccination strategies (Giuntoli et al., 2009; Yigit et al., 2011). Studies of cytokines in the tumour environment are frequently conducted in vitro by addition of biological fluids such as malignant ascites or tumour-conditioned media to cell cultures, while functional characterization of individual proteins is attempted by their selective antibody-mediated neutralization. In our laboratory, we investigate how factors within ovarian carcinoma-associated ascites affect Toll-like receptor (TLR)-mediated activation of monocyte-derived DC. Ovarian carcinoma-associated ascites contains a complex mixture of anti- and pro-inflammatory factors which can differ significantly between individual patients and which has the potential to influence the activation of various cell types (Chen et al., 2009; Mustea et al., 2009; Nowak et al., 2010; Yigit et al., 2010). We observed that ascites partially impairs DC activation in response to TLR agonists in vitro (unpublished data) and we decided to selectively neutralize various factors using specific antibodies to identify the mechanism by which ascites reduces DC activation. IL-10 is one well-known immunosuppressive factor present in ovarian carcinoma ascites and has previously been implicated to play an important role in disease progression and prognosis (Punnonen et al., 1998; Santin et al., 2001; Mustea et al., 2006; Zhou et al., 2007; Giuntoli et al., 2009; Yigit et al., 2011; Matte et al., 2012). We therefore considered IL-10 to be a candidate protein potentially important to the immunosuppressive effects of ascites. Tumour cells, but also tumour-associated antigen-presenting cells have been shown to produce IL-10 and are thought to contribute to the IL-10 levels in ascites (Melichar et al., 1998; Loercher et al., 1999; Zhou et al., 2007).

* Corresponding author at: Biotherapeutics Division, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar EN6 3QG, UK.
E-mail address: sandra.diebold@nibsc.org (S.S. Diebold).

http://dx.doi.org/10.1016/j.jim.2017.01.008

Incidentally, DC produce IL-10 upon TLR activation (Jarrossay et al., 2001; Boonstra et al., 2006). IL-10 induction under pro-inflammatory conditions is an important protective mechanism of the immune system preventing excessive and uncontrolled immune activation. Because of the induction of DC-derived IL-10, blocking experiments based on the addition of neutralizing antibodies to DC cultures cannot conclusively attribute the suppression to ascites-derived paracrine IL-10.

To gain a better understanding of the influence of ascites on DC activation, we explored the relative importance of ascites-derived versus autocrine IL-10 in our experimental system. Our aim was to deplete this protein from ascites before addition of the fluid to cell cultures, allowing us to discriminate between the distinct influences of autocrine versus paracrine IL-10. The establishment of a suitable protocol proved challenging, but we eventually succeeded and present here a method for selective depletion of proteins from ascites. In addition to IL-10, we expanded the application to another immunosuppressive factor, namely PGE_2 (Kalinski, 2012), which we had also identified as a candidate protein in malignant ascites that may partially impair DC activation (unpublished data).

2. Material and methods

2.1. Patient specimens

The collection of ascites for this study was approved by the Guy's Research Ethics Committee (REC Number: 09/H0804/45). Informed consent was obtained from all participants. Ascites from patients suffering from advanced stage (FIGO stage III or IV) high grade serous ovarian carcinoma was collected by paracentesis (St Thomas' Hospital, London, UK). Upon receipt, ascites samples were centrifuged for 10 min at 270*g*, allowing the separation of the fluid into a cellular fraction and non-cellular supernatant. Only the non-cellular supernatant was used in this study. The material was stored in aliquots at −80 °C. Upon thawing, before cytokine depletion and use in cell cultures, the non-cellular fluid was passed through a syringe-driven sterile filter of 0.2 μm pore size (Appleton Woods, Birmingham, UK).

2.2. Neutralizing antibodies

IL-10 neutralizing antibody (clone 25,209; RRID: AB_358066) and the corresponding mouse IgG2b isotype control antibody (clone 20,116; RRID: AB_357344) were from R&D Systems (Abingdon, UK). Neutralizing αPGE_2 antibody (clone 2B5; mouse IgG1; RRID: AB_327973) was from Cayman Europe (Tallinn, Estonia) and the corresponding mouse IgG1 isotype control antibody (clone 11,711; RRID: AB_357346) was purchased from R&D Systems.

2.3. Depletion of IL-10 using protein G-coated agarose beads

IL-10 neutralizing antibody or relevant mouse IgG2b isotype control antibody were added to sterile filtered ascites at concentrations of 5 μg/ml or 20 μg/ml and the samples were incubated for 30 min at 4 °C. After addition of Protein G agarose beads (Thermo Scientific, Cramlington, UK) (100 μl beads per 1 ml of ascites) the samples were incubated for 30 min at room temperature (RT). With a binding capacity of 2 mg of antibody per 100 μl the amount of Protein G-coupled agarose beads exceeded the amount of used antibody by 400× (5 μg/ml) and 100× (20 μg/ml) fold, respectively. Samples were then centrifuged at 420*g* for 5 min, and the supernatant was carefully harvested. Centrifugation at identical speed and duration was repeated to ensure complete removal of residual agarose beads.

2.4. Depletion of IL-10 using protein G coated magnetic beads

For immobilization of αIL-10 antibody or relevant mouse IgG2b isotype control antibody on PureProteome Protein G Magnetic Beads (Millipore, Livingston, UK), the manufacturer's protocol was followed. Briefly, 50 μl Protein G Magnetic Beads were incubated with 10 μg/ml αIL-10 neutralizing antibody or respective mouse IgG2b isotype control antibody for 10 min at RT with continuous mixing. Beads were then washed in 500 μl PBS containing 0.1% Tween 20 with the help of the PureProteome magnetic stand (Millipore). Subsequently, 200 μg chromatographically purified mouse IgG (Invitrogen, Paisley, UK) was added to each sample in order to saturate vacant IgG-binding sites on Protein G Magnetic Beads. Incubation for 10 min at RT with continuous mixing was followed by 3× washing in 500 μl PBS containing 0.1% Tween 20. Antibody-coated Protein G Magnetic Beads were added to 1 ml sterile filtered ascites and incubated for 1 h or overnight at 4 °C with continuous mixing. After incubation, magnetic beads were removed by placing the microcentrifuge tubes into the magnetic stand and transfer of the bead-free ascites into a fresh microcentrifuge tube. This procedure was repeated two more times to ensure complete removal of magnetic beads.

2.5. Depletion of IL-10 or PGE_2 using NHS coated magnetic beads

For conjugation of αIL-10 antibody, αPGE_2 antibody or relevant isotype control antibodies (mouse IgG2b or IgG1) to PureProteome *N*-Hydroxysuccinimide (NHS) FlexiBind Magnetic Beads (Millipore), the manufacturer's protocol was followed. To ensure successful coupling of antibody to the PureProteome NHS Magnetic Beads, the antibodies must be present at a concentration of 2 mg/ml or above. The stock concentration of both isotype control antibodies as well as the αIL-10 antibody was 500 μg/ml and a further concentration was therefore required to reach 2 mg/ml. For this, a defined amount of antibody (100 μg) in solution was pipetted into an Amicon® Ultra-0.5 30 K Device (Millipore), and centrifuged at 14,000 ×*g* for 7 min. The obtained concentrated antibody solution was recovered from the device and the volume was adjusted to 50 μl with PBS, resulting in a final antibody concentration of 2 mg/ml. The αPGE_2 antibody was supplied at a concentration of 2 mg/ml and no further concentration was therefore required.

For the coupling of antibodies to PureProteome NHS Magnetic Beads, 100 μl bead slurry was added to a microcentrifuge tube (Costar® Sigma-Aldrich), and beads were washed 1× with ice-cold equilibration buffer (1 mM HCl; Millipore) with the help of PureProteome Magnetic Stand (Millipore). 50 μl of respective antibody solution (2 mg/ml) was added immediately, followed by incubation for 2 h at RT. Subsequently, beads were washed 5× in the magnetic stand with quench buffer (100 mM Tris-HCl, 150 mM NaCl, pH 8.0; Millipore) followed by an incubation of 2 h at RT in quench buffer. The beads were subsequently washed 4× with wash/coupling buffer (PBS, pH 7.4; Millipore) and finally resuspended in 100 μl of PBS. Antibody-coated beads were stored at 4 °C until used. For the depletion, the αIL-10 or αPGE_2 antibody-coated beads were incubated with ascites from ovarian carcinoma patients (20 μl antibody-conjugated bead slurry per 1 ml ascites) overnight at 4 °C with continuous mixing. To control for the specificity of the depletion, isotype control mouse IgG coated beads were incubated with ascites samples for mock depletion. After overnight incubation, beads were removed from ascites samples using the PureProteome Magnetic Stand. Ascites depleted of IL-10, PGE_2 and mock-depleted samples were stored at −80 °C until their use in activation assays.

2.6. Detection of IL-10 or PGE_2 in depleted ascites samples

To verify successful depletion of IL-10 or PGE_2 from ascites, levels of IL-10 were measured by Flow Cytomix® human IL-10 Simplex Kit (eBioscience, Hatfield, UK). The manufacturer's protocol for Simplex Kits was followed using dilution buffers, reagents and a 96-well filter plate provided in the Flow Cytomix Basic Kit (eBioscience). Samples were transferred into FACS tubes for acquisition on the FACS Canto II (BD), and data was analyzed and quantified using Flow Cytomix® Analysis Software (eBioscience).

For measurement of PGE_2, High Sensitivity PGE_2 Parameter Assay kit (Arbor Assays, Ann Arbor, MI, U.S.A.) was used. The manufacturer's